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13. ABSTRACT (Maximum 200 Words)
Breast cancer is the most common malignancy in women. In U.S., 180,000 new cases are diagnosed and 45,000 deaths occur each year, Current therapy for this disease is aggressive and frequently mulitating. We have been developing a *Listeria monocytogenes*-based Her2/neu vaccine for breast cancer. *L. monocytogenes* has been successfully used as a vaccine vector and tested in several disease models. To improve our immunotherapeutic approach to breast cancer, we are currently investigating the NY-ESO-1 antigen, which is expressed in a large proportion of breast cancers. NY-ESO-1 is the most immunogenic member of the Cancer-Testis antigen family. It is now widely accepted that tumors can escape immunotherapies targeting a single antigen by losing expression of that antigen. In this case, association of Her2/neu and NY-ESO-1 could provide a more efficient vaccine against breast cancer. In this study, we constructed several NY-ESO-1 recombinant *Listeria monocytogenes*. We found that the C-term region of NY-ESO-1, which contains the important HLA-A2/157-165 epitope, is poorly secreted by *Listeria*. We are also generating a NT-2 (her2/neu positive) and 4T1-based breast cancer models in the mouse to test our NY-ESO-1 and Her2/neu vaccines. These recombinant *L. monocytogenes*-based vaccines are a potential therapeutic strategy for breast cancer treatment.

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I. Introduction

Breast cancer is the most common malignancy in women. In U.S., 180,000 new cases are diagnosed each year, in addition to 45,000 deaths caused by breast cancer. Current therapy for this disease (such as surgery, radiation and chemotherapy) is aggressive and in some cases mutilating. More recently, new developments in tumor antigen identification and immune activation raised interest in cancer immunotherapy, which is specific against the cancer cells and with fewer side effects.

The initial goal of this project was to develop a *Listeria monocytogenes*-based vaccine against the Her2/neu antigen, which is overexpressed in a high percentage of breast cancers. As previously reported by Dr. Mary E. Dominiecki last year, several Listeriolysin-O (LLO)-Her2/neu fusion proteins were constructed and confirmed to be expressed and secreted by *Listeria*. The efficacy of these vaccines is now being evaluated in animal models in our laboratory. *L. monocytogenes* has been successfully used as a vaccine vector and tested in several disease models. This gram-positive facultative intracellular bacterium preferentially infects antigen-presenting cells (APC), such as macrophages and dendritic cells (DC), triggering a strong cell-mediated immune response, stimulating both CD8 and CD4 T cells. Most importantly, these responses are extended to the passenger antigen expressed by a recombinant *Listeria* vector (Weiskirch and Paterson, 1999). In fact, vaccination with recombinant *Listeria* expressing target antigens is able to cure mice affected with established tumors, through activation of strong cell mediated immunity (Pan *et al.*, 1995 and 1999, Gunn *et al.*, 2001). A unique characteristic of *L. monocytogenes* is its ability to escape to the cytosol from the vacuole, allowing *L. monocytogenes*-encoded antigens to reach both the MHC class I and class II antigen-presentation pathways.

To improve our immunotherapeutic approach to breast cancer, it is important we increase the scope of breast tumor antigens represented in our *Listeria* vaccines. Although Her2/neu is a potential candidate for breast cancer vaccines, it is a self protein and vaccination regimens have shown only moderate success. In addition, it is now widely accepted that tumors can escape immunotherapeutic strategies that target a single antigen by losing expression of that antigen. A promising group of tumor antigens is the so-called cancer/testis (CT) antigens, which are universally expressed in testis and also in a wide range of different tumors. NY-ESO-1 is a member of the CT antigen family, whose gene encodes for a protein with 180 residues (Chen *et al.*, 1997). Immunohistochemical analysis and mRNA detection of NY-ESO-1 revealed that this antigen is expressed in 20 to 30% of lung, bladder and ovarian cancers and melanoma (Jungbluth *et al.*, 2001). In breast tumors, Sugita *et al.* (2004) detected the NY-ESO-1 mRNA in 42% and 68% of the specimens from malignant and benign tumors, respectively. NY-ESO-1 is the most immunogenic CT antigen described so far and antibodies against it are found in 40 to 50% of the patients with NY-ESO-1-expressing tumors (Stockert *et al.*, 1998). Interestingly, more than 90% of the patients with antibodies against NY-ESO-1 also develop a specific $CD8^+$ T-cell response for this antigen (Jäger *et al.*, 2000). In breast cancers, a higher rate of NY-ESO-1 expression was observed among tumors with high histological grade and negative hormone receptor status, suggesting that NY-ESO-1 could be a potential tumor antigen for immunotherapy in those cases with a poor prognosis (Sugita *et al.*, 2004).

II. Body

Her2/neu and NY-ESO-1 are two potential candidates for immunotherapy of breast cancer, especially in patients with a poorer prognosis. Additionally, it is likely that a combination of these two antigens provides a more efficient therapeutic approach. Furthermore, NY-ESO-1 has some advantages, as a high immunogenicity and expression in normal tissues restricted to testis, although sometimes a low expression can be detected in ovary and placenta.

The first step is to generate recombinant *L. monocytogenes*, which express the NY-ESO-1 gene. Previous results from our laboratory indicates that target antigens fused to a truncated version (441 residues) of the listerial protein LLO are more effective in inducing regression of established tumors in mice (Gunn *et al.*, 2001). We cloned the entire sequence of the NY-ESO-1 gene in frame with the LLO gene of *L. monocytogenes* into the pGG55 plasmid. The expression of the LLO-NY-ESO-1 fusion gene is under the control of the LLO promoter *hly*. We also considered that a fusion protein with the entire NY-ESO-1 sequence might not be properly expressed due to its high hydrophobic content. Therefore, in addition to the entire NY-ESO-1 gene (residues 1 to 180), we also cloned partial overlapping sequences of the gene, encoding for: 1) residues 1 to 108; 2) residues 101 to 156; 3) residues 101 to 180 and 4) residues 148 to 180 (figure 1).

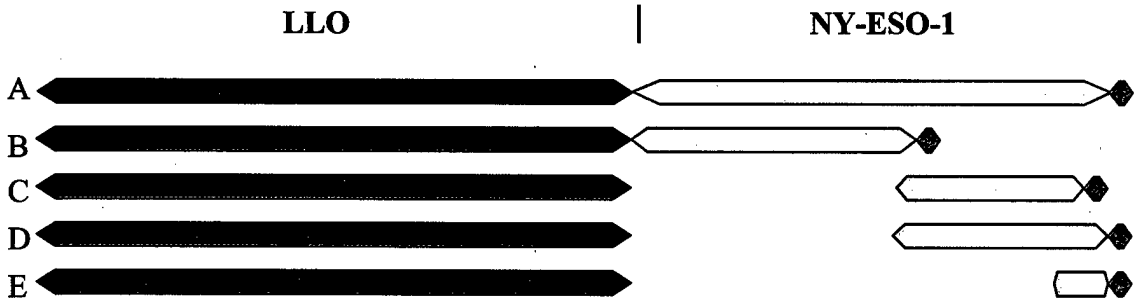

Figure 1. First LLO_NY-ESO-1 fusion proteins constructed and tested for expression and secretion in *Listeria monocytogenes*. A) LLO_NY-ESO-1_1-180; B) LLO_NY-ESO-1_101-108; C) LLO_NY-ESO-1_108-156; D) LLO_NY-ESO-1_101-180; E) LLO_NY-ESO-1_148-180. All constructs have the FLAG epitope in the C-term region.

The recombinant *Listeria* were passaged twice in mice for *in vivo* selection of viable bacteria. The LD_{50} determination showed that the LLO_NY-ESO-1 recombinant Listeria are highly attenuated ($LD_{50}=2.5x10^9$). Analysis of secretion of the recombinant proteins by Western-blot revealed that only the constructs LLO_NY-ESO-1_1-108 and 101-156 were strongly secreted, whereas the construct LLO_NY-ESO-1_1-180 had a weak secretion. On the other hand, the fusion proteins LLO_NY-ESO-1_101-180 and 148-180 could not be detected (figure 2). Expression results show that LLO_NY-ESO-1 fusion proteins containing the C-term region of NY-ESO-1 are poorly secreted. One possible cause is the high hydrophobicity of this region. New constructs were made in an attempt to improve the secretion of a fusion protein containing the C-term portion of NY-ESO-1, given that an important HLA-A2-restricted epitope is located between the residues 157 and 165. Initially, this epitope was cloned into the position 40 of NY-ESO-1 fragment 1 to 108 (LLO_NY-ESO-1_1-108/157-165). A codon-optimized NY-ESO-

1_148-180 fragment was also constructed and cloned into different positions of the LLO protein (positions 178 or 312). Additionally, the C-tem region of NY-ESO-1 protein (residues 101 to 180) was cloned between LLO and the N-term region of NY-ESO-1 (residues 1 to 108) (LLO_NY-ESO-1_101-180/1-108). Unfortunately, none of these new constructs resulted in better secretion of NY-ESO-1 C-term region by *Listeria*, compared to the LLO_NY-ESO-1_1-180 construct. One possible explanation is that the expression of the whole protein is important for the folding of NY-ESO-1 C-term region, avoiding an extensive degradation of this portion of the protein. We are currently testing the ability of some of these constructs (LLO_NY-ESO-1_1-180, LLO_NY-ESO-1_101-180/1-108 and LLO_NY-ESO-1_1-108/157-165) in inducing an immune response against the NY-ESO-1 157-165 epitope. These experiments are being carried out in collaboration with Dr. Gerd Ritter (Ludwig Institute for Cancer Research, New York). We are still exploring new possibilities to get the NY-ESO-1 region better secreted by *Listeria*.

The second step is to generate mouse tumor cell lines that constitutively express the human NY-ESO-1 gene. We chose the 4T1 cell line, which is a cell line derived from a BALB/c mammary carcinoma. The 4T1 mammary carcinoma model is also very useful to evaluate the effect of the vaccine in a metastatic breast disease, since these cells spontaneously metastasize (Pulaski and Ostrand-Rosenberg, 1998). We used a retroviral-based system to transduce this cell line with the human NY-ESO-1 gene, whose expression in this model is under the control of the CMV promoter. However, after some weeks the NY-ESO-1 expression was highly down-regulated in this cell line and the protein cannot be detected (figure 4). This is likely to be due to a down-regulation of the CMV promoter in these cell lines, which has also been described in other models (Gill *et al.*, 2001). To overcome this difficulty, we are replacing the CMV promoter in the retrovirus plasmid by the human Ubiquitin C (hUbC) promoter, which is constitutively active in mammalian cells for periods as long as 6 months. The stability of the UbC promoter makes it a better choice for long-term studies in animal models. We have amplified and cloned the human UbC promoter from a commercially available plasmid. However, the sequence of our clones is slightly different from the published sequence (GeneBank D63791). Interestingly, these mutations were always the same and were independent of the PCR cycle or the polymerase used. Even after using a proof-reading polymerase (Pfu Turbo, Stratagene), we observed the same mutations. However, after sequencing our template, we found the same previous sequence, confirming the difference from the published sequence. Now, we have cloned the hUbC promoter into the lentivirus vector to verify its performance.

We will also extend our model to the NT-2 cell line, which is derived from a spontaneously arising mammary carcinoma in ratHer2/neu transgenic FvB mouse. Our plan is to transduce this cell line with NY-ESO-1 and evaluate the efficacy of our vaccines in this model, comparing the Her2/neu and NY-ESO-1 immunotherapies, besides a possible combination of both. Initially, tumor modeling studies will be carried out to establish growth kinetics. After, mice will be given a dose of tumor cells enough to establish a lethal tumor in untreated animals in a few weeks. When the tumor reaches a palpable size (4-5 mm in diameter), mice will be given a dose of NY-ESO-1 recombinant *L. monocytogenes*, with additional boosting doses at regular intervals. To evaluate the vaccine efficacy, several variables will be analyzed in untreated and treated mice, including tumor size and progression, the frequency of $CD4^+$, $CD25^+$ and $CD8^+$ T cells in splenocytes and tumor infiltrating lymphocytes, secretion of cytokines, and activation and cytotoxicity of $CD8^+$ cells against the NY-ESO-1-expressing tumor cell lines (Gunn *et al.*, 2001).

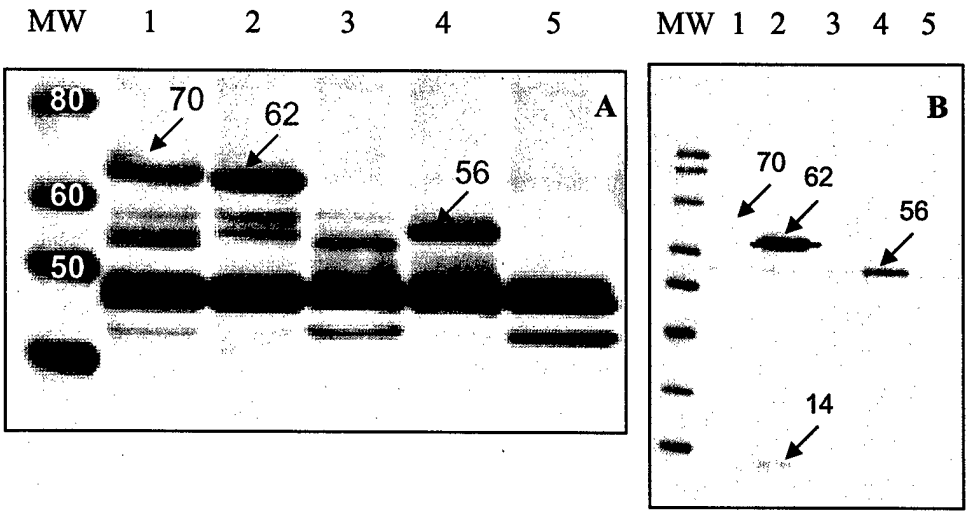

Figure 2. Western-blot of LLO_NY-ESO-1 constructs. Proteins from bacterial culture supernatants were precipitated in 10% trichloroacetic acid. Primary antibody: (A) polyclonal anti-LLO; (B) monoclonal anti-FLAG. MW: molecular weight marker; lane 1: LLO_NY-ESO-1_1-180 (~70KDa); lane 2: LLO_NY-ESO-1_1-108 (~62KDa); lane 3: LLO_NY-ESO-1_101-180 (~58KDa); lane 4: LLO_NY-ESO-1_101-156 (~56KDa); lane 5: LLO_NY-ESO-1_101-148 (~54KDa). In (A), a band of 48KDa, corresponding to the truncated LLO, is observed. In (B), a band of 14KDa, corresponding to the cleaved off NY-ESO-1_1-108 protein, is observed.

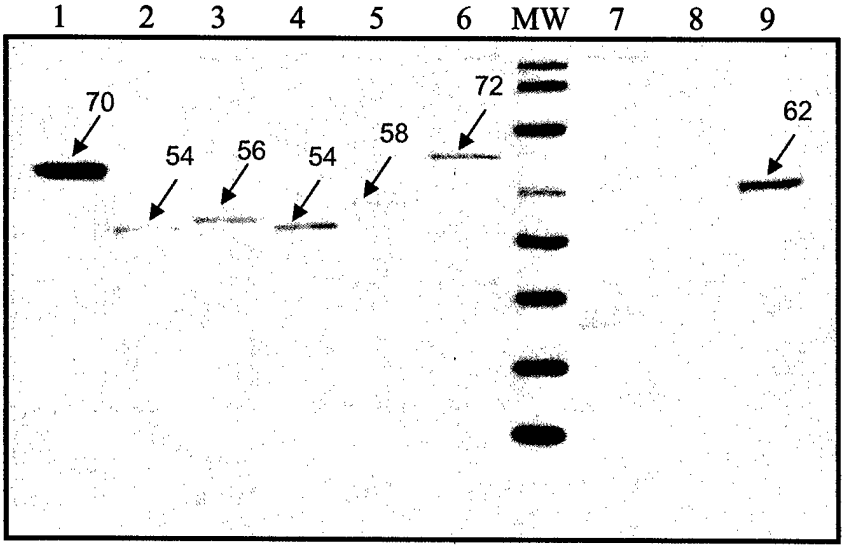

Figure 3. Western-blot analysis of constructs carrying the C-term region of NY-ESO-1 fused to LLO and secreted by *L. monocytogenes*. Primary antibody: anti-FLAG. MW: molecular weight marker; lane 1: LLO_NY-ESO-1_1-180 (~70KDa); lane 2: LLO_NY-ESO-1_148-180 (~54KDa); lane 3: LLO_NY-ESO-1_148-180_GATEWAY (~56KDa); lane 4: codon-optimized LLO_NY-ESO-1_148-180 (~54KDa); lane 5: LLO_NY-ESO-1_101-180 (~58KDa); lane 6: LLO_NY-ESO-1_101-180/1-108 (~72KDa); lane 7: LLO(312)_NY-ESO-1_148-180 (~54KDa); lane 8: LLO(178)_NY-ESO-1_148-180 (~58KDa); lane 9: LLO_NY-ESO-1_1-108/157-165 (~62KDa).

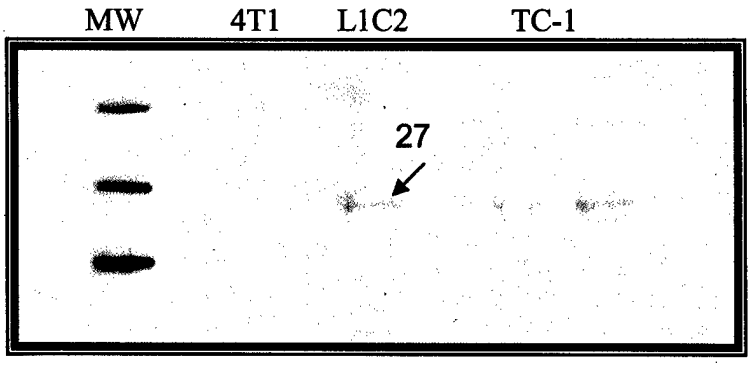

Figure 4. Western-blot analysis using the ES121 monoclonal antibody (anti-NY-ESO-1). MW: molecular weight marker. L1C2 and TC-1 are derived from lung carcinoma and lung epithelial cells, respectively, and were also transduced with NY-ESO-1 gene. The 27KDa band corresponds to the NY-ESO-1/V5 epitope protein.

III. Key Research Accomplishments

- Construction of several LLO_NY-ESO-1 fusion proteins for *Listeria*;
- Analysis of expression and secretion of each fusion protein by Western blotting;
- Passaging in mice of LLO_NY-ESO-1_1-180, 1-108, 101-156, 1-108/157-165, 101-180/1-108;
- Determination of LD50 for LLO_NY-ESO-1_1-108, 101-156 and 1-108/157-165;
- Cloning of NY-ESO-1 in a lentivirus vector and generation of a lentivirus particles;
- Transduction of 4T1 cells with NY-ESO-1 recombinant lentivirus and analysis of NY-ESO-1 expression;
- Amplification and cloning of the human Ubiquitin C promoter.

IV. Reportable Outcomes

This work was presented in October 2003 in the *Cancer Vaccines 2003 – Cancer & HIV Vaccines: Shared Lessons*, sponsored by the Cancer Research Institute and the Ludwig Institute for Cancer Research, held in New York, USA

V. Conclusions

In summary, we constructed several recombinant *Listeria monocytogenes* that express NY-ESO-1. We found that the C-term region of NY-ESO-1, which contains the HLA-A2 157-165 epitope, is poorly secreted by *Listeria*. Additionally, we are trying to generate a 4T1 and NT-2 based breast cancer models in the mouse to test our NY-ESO-1 vaccine. NY-ESO-1 is a potential candidate for generic cancer vaccination and like Her2/neu, it is expressed in a large proportion of breast cancers. Although clinical trials using a NY-ESO-1 peptide-based vaccine have generated promising results, improvement of the vaccine approach is needed. Vaccination with NY-ESO-1 recombinant *L. monocytogenes* is a potential strategy that will be tested in clinical trials and compared to other vaccine approaches, such as utilization of viral vaccine constructs, different adjuvants and dendritic cell activators. Furthermore, concomitant vaccination with NY-ESO-1 and Her2/neu is likely to provide a better therapeutic approach for breast cancer.

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